Fiber composite slices for multiplexed immunoassays

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Fabrication methods for the development of multiplexed immunoassay platforms primarily depend on the individual functionalization of reaction chambers to achieve a heterogeneous reacting substrate composition, which increases the overall manufacturing time and cost. Here, we describe a new type of low-cost fabrication method for a scalable immunoassay platform based on cotton threads. The manufacturing process involves the fabrication of functionalized fibers and the arrangement of these fibers into a bundle; this bundle is then sectioned to make microarray-like particles with a predefined surface architecture. With these sections, composed of heterogeneous thread fragments with different types of antibodies, we demonstrated quantitative and 7-plex immunoassays. We expect that this methodology will prove to be a versatile, low-cost, and highly scalable method for the fabrication of multiplexed bioassay platforms. © *2015 AIP Publishing LLC*. [http://dx.doi.org/10.1063/1.4927590]

INTRODUCTION

For many diagnostic, drug development, and biomedical research applications, the simultaneous analysis of large numbers of biomolecules in small sample volumes with high-throughput has traditionally been accomplished using novel technologies, such as surface-based microarrays[1–9] and bead-based suspensions.[10–17] These platforms adopt miniaturized, multiplexed and parallel processing and detection methods, and several technologies that immobilize surface binding molecules, such as DNA[18–22] or protein,[6–9,23,24] have been successfully developed. The platforms developed to date have achieved reductions in cost while excluding complex instrumentation for multiplexed bioassays.

However, for inexpensive commercial kits and point-of-care diagnostics, platforms that utilize cheap and widely available materials such as cotton, paper, and thread have been proven to be more desirable. It has been shown in many previous works that capillaries in these fiber meshes enables pump-less lateral flow that can replace conventional lab-based microfluidic systems involving soft lithography and pump devices. In case of thread platforms, various circuit functions such as fluid splitting, merging, and mixing were demonstrated as well as immunoassays.[25–32] Recently, Zhou *et al.*[33] demonstrated a colorimetric immunoassay on threads and provided evidence that thread platforms are capable of quantitative high-sensitivity assays. Noticeably, they performed multiplexed reactions by knotting the thread channels together. This multiplexing capacity is an important factor for cost-effective and time-efficient

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assay and is usually preferred to be maximized. A number of reports introduced inexpensive fabrication methods that enable multiplexed assays using hydrophobic-hydrophilic patterned cotton,[34] craft punch patterned paper,[35] or wax-printed paper.[36] However, previous methods for multiplexing thread immunoassays have potential concerns regarding reproducibility and flow uniformity when increasing the multiplexing number, or in other words, the number of woven threads per knot. Conclusively, a more scalable multiplexing method would be desirable.

Herein, we report a simple and scalable method for high-throughput fabrication of multiplexed bioassay platforms. In this platform, a particle-like array is composed of multiple assay compartments made of fiber. Each assay compartment contains fibers functionalized with capture antibodies against target analyte to be quantified, thus a fiber-based multiplexed immunoassay. The fabrication process involves antibody-functionalizing individual fiber, arranging multiple types of functionalized fibers into a bundle, and slicing the bundle into sections. These sections are particle-like arrays containing multiple assay compartments and thus enable multiplexed immunoassay on cheap fiber matrices. Among many types of fiber matrices, we chose cotton threads because they are cheap, widely available, compatible with many biological materials, and have dense micro-capillaries desirable for surface chemistry.[25–31] In this meso-scale particle-like array, individual spots are covered with distinct probes by selective surface-modification. Also, the number, size, and arrangement of separate spots on an array are readily designable during the bundling process to meet the user's specifications. Using this platform, we performed cytokine profiling with seven cytokines in a singleplex and multiplex manner.

EXPERIMENT

Materials and reagents

Reagents including Bovin serum albumin (BSA), (3-aminopropyl)triethoxysilane (APTES) > 98%, Succinic anhydride (SA) > 99%, and Triethylamine (TEA) > 99.5% were purchased from Sigma Aldrich. Other chemicals and solvents including N-Hydroxysulfosuccinimide Sodium (NHS) salt 97%, Absolute ethanol, NN-Dimethylformamide (DMF), and 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) 98+% were purchased from Alfa Aesar. Capture antibodies (cAb), biotin-labelled polyclonal antibodies (dAb), antigens (Ag), and Alkaline phosphatase (ALP), were purchased from either Millipore, eBioscience, or BD Bioscience. Specifically, immunoassay sets (capture antibody, antigen, and detection antibody) for IL-1β, IL-2, IL-5, IL-10, and IL-17A were purchased from eBioscience. Immunoassay set for IL-7, capture antibody and detection antibody for IL-12 were purchased from BD Bioscience. IL-12 antigen was purchased from Millipore. Product catalog numbers for IL-1β, IL-2, IL-5, IL-7, IL-10, IL-12, and IL-17A, respectively are as follows. Capture antibody:14-7018, 14-7029-85, 14-7052-85, 554493, 14-7108-85, 555065, and 14-7178-85; antigen: 14-8018-80, 14-8029-81, 39-8059-65, 554608, 14-8109-80, IL029, and 14-8179-80; detection antibody: 13-7016-85, 13-7028-85, 13-7059-85, 554494, 13-7109-85, 554660, and 13-7179-85. Alkaline phosphatase substrate (alkaline phosphatase blue) was purchased from Sigma Aldrich. Streptavidin-linked phycoerythrin (SA-PE) was purchased from ProZyme. [2-(N-morpholino)ethanesulfonic acid] (MES) buffered saline pack was purchased from Thermo Scientific. Chemicals sensitive to moisture, such as NHS and EDC, was stored in desiccators. Other reagents were stored according to the manufacturer's recommendation.

Preparing capture-antibody cross-linked threads

Locally purchased (Dongdaemun shopping complex, Seoul, Korea) 100% pure cotton threads (cellulose fiber diameter 10–30 μm, thread diameter 625 μm, bleached, no mercerization) were first treated with O_2 plasma and washed with absolute ethanol to introduce more hydroxyl groups. To our experience, commercial cotton threads that were treated with preservative chemicals showed lower cross-linking quality. Also since threads dyed with colours frequently contained unwanted autofluorescence, we only used these as coding threads. Then for surface amine functionalization threads were mixed with 0.095 g/ml APTES solution

(prepared in absolute ethanol) and stirred at 25 °C for 2 h. Threads were washed with absolute ethanol, dried at 110 °C for 10 min, and cooled back to ambient temperature. It is important to fully dry the threads before further modification. Terminal amine carboxylation was done by adding carboxylation solution (6 mg SA, 8.4 μl of 7.2M TEA in 1 ml DMF) and stirring at 25 °C for 2 h. Threads were sequentially washed with DMF, absolute alcohol, and MES buffered saline (MES). EDC/NHS-cross-linking solution (5 mg NHS, 5 mg EDC per 1 ml MES) was then added and stirred at 25 °C for 25min followed by washed with MES. We then spread the threads on a flat surface and submerge in 14 μg/ml capture antibody solution (prepared in MES). Finally, threads were refrigerated at 4 °C for solvent evaporation and were either stored without further modifications or wrapped into bundle particles. Depending on humidity condition of the laboratory, solvent evaporation can be accelerated by placing the antibody solution-submerged threads in a mild-condition desiccator. After antibody cross-linking, threads were incubated with a blocking solution (1% BSA in PBS) for 2 h at 25 °C to block remaining functional groups and prevent non-specific protein binding. The entire time required for producing antibody-linked threads is approximately 8 h.

Fluorogenic reporter-based immunoassay

Capture antibody cross-linked threads or bundle particles were incubated with concentration-defined antigen prepared in dilution solution (0.1% BSA in PBS) and stirred at 4 °C for 2–16 h. After washing with washing solution (0.1% BSA, 0.02% Tween 20 in PBS), threads were incubated with 4 ng/ml biotin-labelled anti-antigen polyclonal antibodies (prepared in dilution solution) at 25 °C for 1 h and washed with washing solution. Finally, threads were incubated with SA-PE solution (1 μg/ml SA-PE prepared in dilution solution) for 30 min and were washed rigorously with washing solution before imaging. We imaged with a 4× lens on an Olympus microscope and fluorescence emission wavelength filtered at 575 nm–625 nm. After image stitching, fluorescence intensities were quantified using Image J.

Colorimetric immunoassay

Capture antibody cross-linked threads or bundle particles were incubated with concentration-defined antigen prepared in dilution solution (0.1% BSA in PBS) and stirred at 4 °C for 2–16 h. After washing with washing solution (0.1% BSA, 0.02% Tween 20 in PBS), threads were incubated with 4 ng/ml biotin-labelled anti-antigen polyclonal antibodies (prepared in dilution solution) at 25 °C for 1 h and washed with washing solution. This was followed by incubation in 5 μg/ml ALP solution (prepared in dilution solution) at 25 °C for 30 min. Threads or bundle particles were then submerged in 100 μl of ALP substrate (prepared as the manufacturer's description) for precisely 8 min. Right after incubation, they were put on a flat surface and imaged under a loupe. For clear imaging, it is preferable to put the bundle particle on a non-gloss black colored surface.

Single thread fluorogenic immunoassay

We conducted a fluorogenic-reporter based immunoassay on single strands of threads and confirmed positive reactions occurring selectively in subsets containing every reaction required for the assay. The reactions required for the assay are (i) cross-linking of capture antibody, (ii) antigen treatment, (iii) biotin-labelled polyclonal antibody treatment, and (iv) phycoerythrin reporter binding. As shown in Figure S1 in the supplementary material,[37] when the antibody-antigen pair matched, the fluorescence signal was detected. By this experiment, we were also able to exclude any potential false-positive immunoassay results caused by non-specific molecule binding to the chemically modified thread surface.

RESULTS

Fabrication of particle-like array platform

Figure 1(a) shows the concept and a schematic of our platform. One unique feature of this platform is the process for structuring differently functionalized fibers, which involves binding the functional threads together, cladding the thread bundle, and slicing the bundle into small particles. This structuring process is adaptable to automation and allows high-throughput fabrication of identical particle-like arrays without complex instrumentation. Micro-capillaries inside a thread fragment not only provide the substrate for thread functionalization and the subsequent biological reactions but they also hold solution containing biological materials within the cavities, which enables the reaction to occur within a confined volume. Therefore, by handling a single piece of thread composite in a conventional tube, we can perform multiplexed bioassays with low reaction volume. The detection signals can also be observed by the naked eye or using a simple camera.

Characterization of threads as immunoassay substrate

Individual thread strands can be functionalized by physical adsorption or chemically cross-linking the proteins onto the surface of the cellulose microfibers that compose the strand. Physical adsorption only requires simple soaking followed by complete drying to attach proteins. Chemical immobilization links proteins onto the thread through the chemical modification of the cellulose surface of the cotton thread. We compared both immobilization methods by applying detection antibodies tagged with fluorogenic markers and compared their fluorescence intensities. As shown in Figure 2(a), antibody immobilization through the chemical method produces a more stable and sensitive signal for thread-based immunoassays, and we chose this method to functionalize substrates for the following demonstrations.

Next, we investigated the signal intensity variations on different parts of the functionalized thread. The quality of individual particle-like arrays depended on the stability of the functionalization of the thread strand because a sliced fragment of the strand becomes an immunoassay

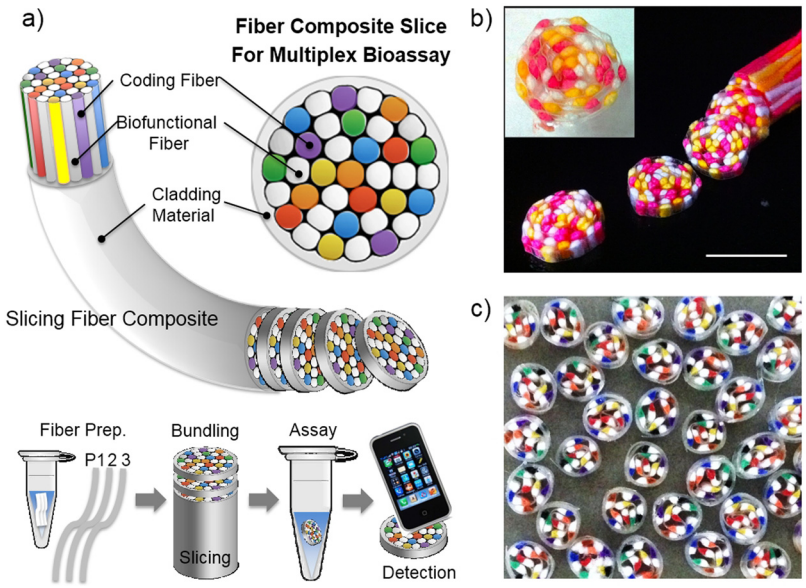

FIG. 1. Conceptual schematic of a particle-like array platform. (a) A single slice is composed of functional fibers, coding fibers, and cladding material. The entire fabrication process for this multiplexed assay platform includes preparing biologically modified fibers and manufacturing the platform by slicing the fiber composites. The multiplexed assay is performed in a single tube, and the signal can be easily detected by the naked eye or using a camera. (b) and (c) The cladded fiber composite is sliced to form a particle-like array for multiplexed bioassays. This fabrication strategy provides a simple way to produce identical platforms in a high-throughput manner (scale bar: 1 cm).

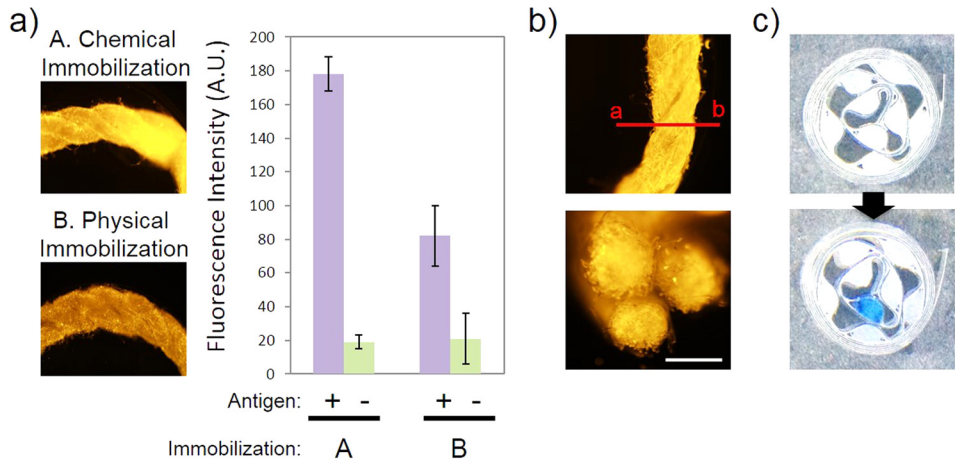

FIG. 2. Characteristics of threads used as immunoassay substrate. (a) Antibody-coated thread fragments are the immunoassay substrate. Comparisons between physical and chemical immobilization show that antibodies were more densely coated on threads when using the chemical cross-linking method. This cross-linking increased the detection sensitivity of this platform. Error bars indicate standard deviations. (b) Antibodies are stably and uniformly immobilized both on the outer surface and inner micro-cavities (scale bar: 0.2 mm). (c) On this thread-based immunoassay substrate, both fluorescence and colorimetric immunoassays can be conducted. The target thread fragment in the slice changes its color to blue due to the enzymatic reaction.

reaction spot on the particle-like array. The detection antibodies tagged with fluorogenic markers were applied to a functionalized thread strand, and the test assay results, shown in Figure 2(b), confirmed that the proteins were successfully immobilized both on the outer surface of the thread and the inner mesh of micro-capillaries through a chemical cross-linking process. This showed that the cross sections of these threads are appropriate sites for reading out fluorescent immunoassay results. In effect, creating an array of these thread cross-sections could be an easy and scalable method to create a cheap multiplexed assay, unless cross reaction or off-target binding occurs.

Confirmation of resistance to antigen off-target binding

If antigens could bind with threads treated with mismatching capture antibodies or randomly bind on threads without specificity, detectable fluorescent signals would appear off-target. To investigate the possibility of antigen off-target binding in our bundle particle assay platform, we conducted a fluorogenic reporter-based immunoassay on bundle particles. We prepared assay particles made up of 6 different kinds of threads each cross-linked with one of 6 capture antibodies; anti-IL-2, IL-4, IL-7, IL-10, IL-12, and IL-17 antibody. We then performed fluorescent immunoassay on these assay particles by first mixing with one of the six antigens (10 ng/ml in 1% BSA-PBS) and incubating for 2 h at 4 °C. The particles were then washed to remove unbound antigens, mixed with polyclonal anti-antigen antibodies (biotinylated, 10 μg/ml in 1% BSA-PBS), and incubated for 1 h at 25 °C. After washing, fluorogenic marker-linked streptavidin (5 μg/ml in 1% BSA-PBS) was applied to the particles for half an hour at 25 °C, and the fluorescent signal was detected using a fluorescence microscope after a harsh washing process. As shown in Figure S2 in the supplementary material,[37] no recognizable off-target fluorescence appeared in any set we tested. Additionally, we investigated off-targeting in higher or lower antigen concentration. Assay particles targeting IL17 were prepared and treated with IL17 at different concentrations during the immunoassay process. As expected, only signals from on-target spots varied in an antigen concentration-dependent manner within a 3 dynamic range (0.1–100 ng/ml) of IL17 concentration (Fig. 3).

These results indicate that multiplexed immunoassay can be performed on this bundle particle platform without significant non-specific or off-target signalling. To describe the advantage of this platform, whereas only one reaction per sample was available for conventional ELISA,

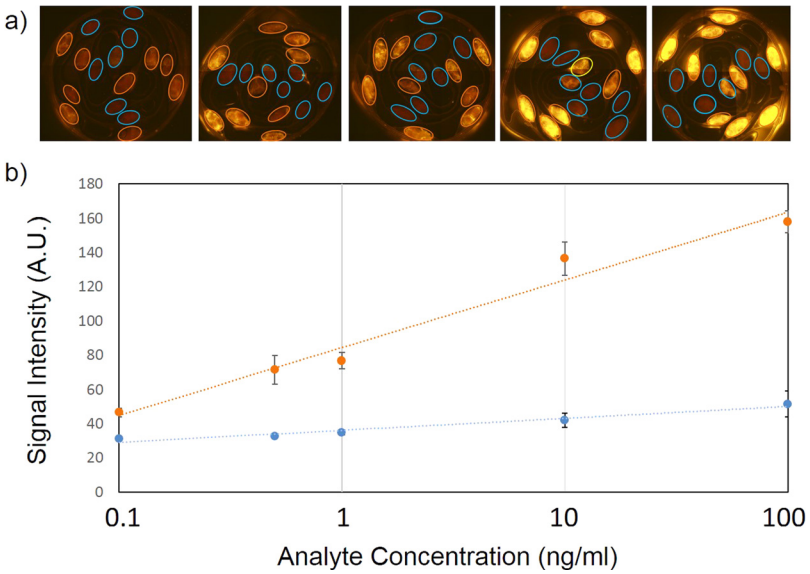

FIG. 3. The fluorescence signal intensity according to the applied IL17 antigen concentration. (a) From left to right, images correspond to IL17 concentration of 0.1 ng/ml, 0.5 ng/ml, 1 ng/ml, 10 ng/ml, and 100 ng/ml. Sections indicated with orange edges are IL17 on-target sites and blue edges indicate non-target control sites. The exposure time was 0.5 s. (b) Plot of the quantified fluorescence signal from the target threads (orange) and non-target threads (blue).

it now becomes possible to read-out multiple immunoassay results from each sample. This enables to quantify various analytes in a single test while decreasing total sample volume required. Consumption of expensive reagents such as capture antibodies and detection antibodies can be reduced proportionally to the number of multiplexing.

Singleplex colorimetric immunoassay

To explore the potential of this platform, we performed immunoassays for seven types of interleukins: IL1β, IL2, IL4, IL6, IL7, IL10, and IL17. These interleukins play several roles in the human body, such as hormonal regulation or molecular signaling. The fiber bundle slice was designed to contain one control fiber and seven protein-immobilized fibers paired with corresponding color-coding fibers, as shown in Figure 4(a). The fabrication process was very convenient: ordered pairs of capture antibody-immobilized threads and color coding threads were placed on the surface of scotch tape and covered with another layer of scotch tape. We rolled the strip of bio-functional threads and cut it into thin thread composite slices with an average thickness of less than 1 mm. The diameter of a slice was approximately 3 mm, depending on the number of fibers in the composite.

On this particle-like array platform, we performed fluorescent immunoassays. A single slice was put into a 1.5 ml tube and the antigen-containing solution, which was diluted to 10 ng/ml in 1% BSA-PBS, was applied to the slice for 2 h at 4 °C. The slices were washed to remove unbound antigens. The enzyme-linked secondary antibodies (10 μg/ml in 1% BSA-PBS) that bind to the specific antigen were added to the thread for 1 h at 25 °C, and unbound antibodies were washed out. For the fluorescent immunoassay, fluorogenic marker-linked protein (5 μg/ml in 1% BSA-PBS) was applied to the thread for half an hour at 25 °C, and the fluorescent signal was detected using a fluorescence microscope after a harsh washing process.

For the colorimetric immunoassay, the procedure starting from raw cotton thread preparation up to capture antibody treatment was the same as fluorogenic reporter-based immunoassay. The difference starts from the bundling process of these threads where color-coded threads were assigned for each cross-linked thread. For this, one color thread was attached adjacent to each cross-linked thread. Therefore, the assay type for each thread can be intuitively decoded. After slicing the resulting bundles into particles, alkaline phosphatase-conjugated protein (5 μg/ml

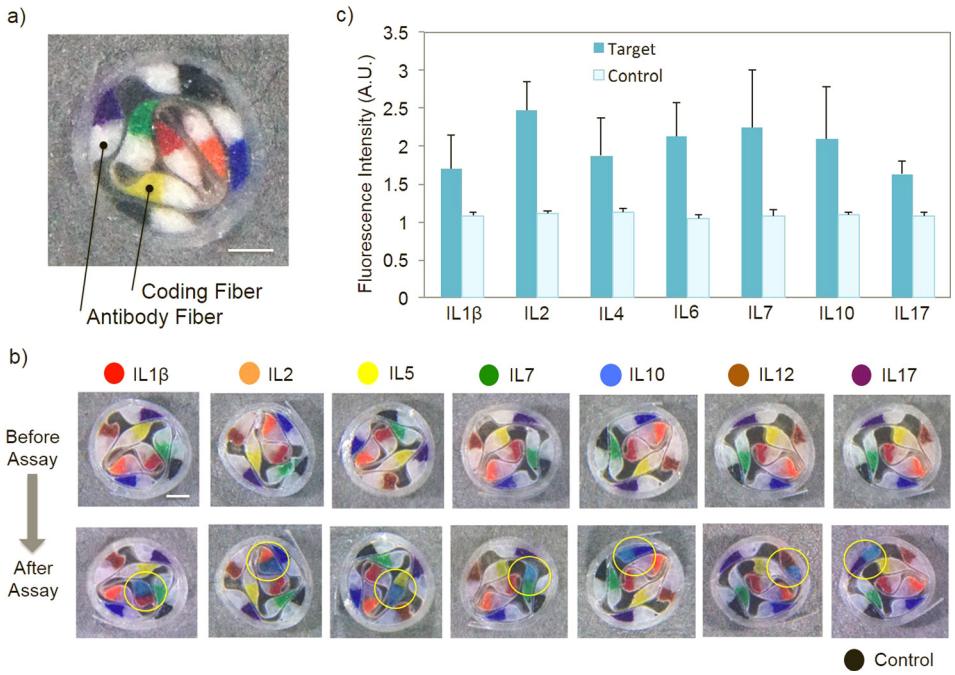

FIG. 4. Singleplexed immunoassay. (a) A pre-defined particle-like array comprising eight types of fibers with adjoining coding fibers. Seven of them are coated with a specific antibody, and the remaining one is a control (BSA coated). The target cytokine can be easily identified by the color code next to the signal. Red, orange, yellow, green, blue, brown, violet, and black threads represent IL1β, IL2, IL5, IL7, IL10, IL12, IL17, and BSA coated fragments, respectively. Scale bar is 1 mm. (b) The target thread fragment (indicated by yellow circle) turns blue after the assay procedure which was discernable compared to the color change of the control thread fragment (black color code). (c) The relative color intensity of the target thread fragment compared to the BSA coated control thread fragment (N = 7). Blue bars: treated with 10 ng/ml of target cytokine. Light-blue bars: treated with PBS (0.1% BSA). Average CV of the seven blue bars was 23.8%.

in 1% BSA-PBS) was applied to the particles for half an hour at 25 °C and then washed out. Then, the substrate of ALP was added and was enzymatically converted into a colored molecule. The colorimetric signal was detectable by the naked eye or by using a camera. The entire colorimetric assay process took approximately 3.5 h for a single procedure. The total reagent volume consumed for a single 7-plex immunoassay was 200 μl including diluted antigen, secondary antibody, and alkaline phosphatase conjugate solution. Next, we applied the substrate solution, which was enzymatically converted into a blue color and, after a few minutes, detected the color change of the fiber fragments in the slice by the naked eye or using a cell phone camera. For this final enzymatic detection process, we analyzed the time profile of color signal intensity (Fig. 5). The color signal saturated after 8 min of enzyme reaction and was selected as our optimum reaction time. Reaction time much longer than this led to diffusion of the blue color into the entire solution and cross-contamination.

Enzyme based colorimetric immunoassay tests rely on the interaction between the immobilized enzymes and the free-floating substrates. In conventional ELISA, substrates that have changed their morphology diffuse through the chamber, intensifying the overall color of the liquid. As a consequence, the enzyme products are significantly diluted and each reaction requires unnecessarily large volume of substrate and enzyme reaction time to produce a visually discernable color signal. In comparison, each thread fragment in this platform is composed of a dense and intertwined network of cellulose micro-capillaries which hinders the diffusion of the morphologically altered substrates out of the threads into the buffer. This significantly increased sensitivity and reduced enzyme reaction time. Additionally, we assume that wrapping thread fragments in impermeable scotch tape and taking a slice prevents substrate permeation into adjacent fragments. As a result, we were able to collocate multiple ELISA reactions on a single particle without significant cross-contamination.

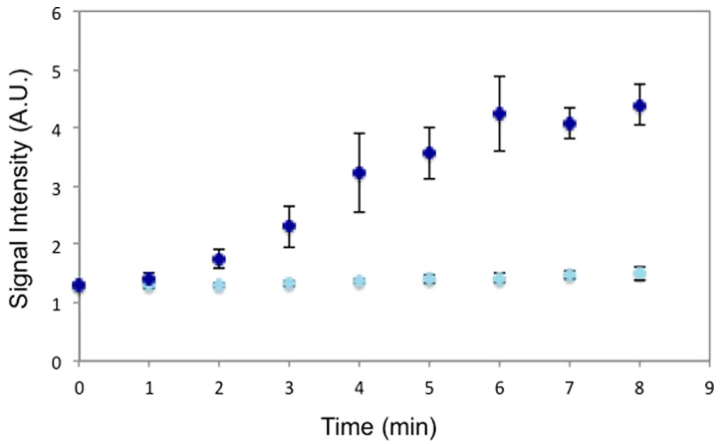

FIG. 5. The change of signal intensity after ALP substrate application. We applied the substrate solution that is converted by enzyme into a blue color and detected the color change of the fiber fragments in the slice using cellphone camera through a loupe. The target thread fragment in the slice changes its color to blue due to the enzyme reaction. The fiber component in a slice is immobilized with IL10 antibody and 10 ng/ml of IL10 was applied to the sample. Blue dots represent the binding signal and the sky-blue dots represent the control signal (N = 54).

As shown in Figure 6(a), simple quantitative assays were conducted to widen the utility of our platform. We obtained the working range of our platform against three cytokines: IL1β, IL10, and IL17. Although the overall signal intensity varied among different cytokines, the working ranges were similar; approximately 100 pg/ml to 10 ng/ml. The decrease in signal when antigen concentration was higher than 10 ng/ml may probably resulted from high dose hook effect that can cause false-negative in immunoassay.

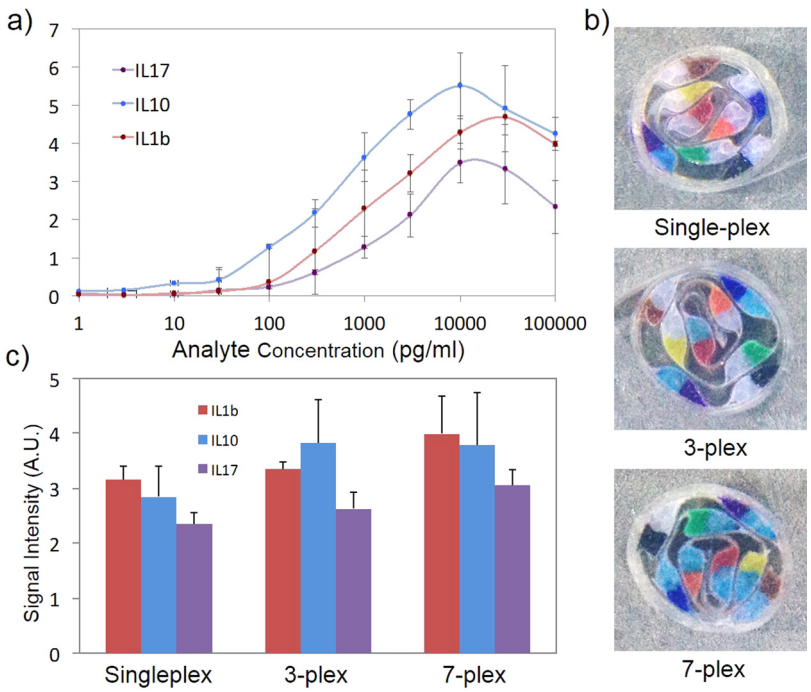

FIG. 6. Multiplexed and quantitative immunoassays. (a) The signal intensities gradually increase with antigen concentrations for three types of cytokines: IL1β, IL10, and IL17. The error bar is the standard error from five independent experiments. (b) Singleplex, 3- and 7-plex immunoassays were performed on the particle-like microarrays. Target thread fragments show clear color change after the assay procedure. (c) The overall tendency of signal intensity is maintained as the multiplexing number of cytokines increases. Error bars are the standard error from three independent experiments.

Multiplex colorimetric immunoassay

We then explored the potential of this platform for multiplexed assays by performing 3- and 7-plex immunoassays (Fig. 6). For this, we utilized seven cytokine batches that were verified to have no cross-reactivity in the previous singleplex experiments. All antigens, 10 ng/ml each, were mixed together and incubated with an assay particle containing the corresponding anti-antigen threads. After the immunoassay procedures, the results of 3- and 7-plexed assays were successfully decodable as shown in Figure 6(b). The graph shown in Figure 6(c) indicates a discrepancy between the single, 3- and 7-plex assay results, which might be attributed to non-specific binding and physical adsorption on the surface of the micro-capillaries in the thread fragments; however, the signal intensities between the singleplexed and multiplexed assays are not significantly different. Our current assay protocol takes up approximately 4 h, which is suitable for lab tests but yet impractical for point-of-care applications. We presume that incubation times can be further reduced by optimizing the capture antibody crosslinking process and adopting a more hydrophilic bundling material.

CONCLUSION

In this paper, we introduced a new type of manufacturing process for the development of particle-like arrays for a multiplexed bioassay platform using individually functionalized thread strands. This process, which includes bundling and cutting of the functionalized thread substrates, establishes a versatile and potentially high throughput method for the fabrication of multiplexed bioassay platforms with controllable architectures and functionalities. Thread fragments contain micro-capillary networks that provide volumetrically abundant sites for chemical modification and antibody cross-linking. To demonstrate this, we modified the cellulose fibers in pure cotton threads in order to chemically cross-link capture antibodies and showed that immunoassay signal intensity increased compared to simple antibody adsorption. The capillary network in threads also hinders enzyme reaction products from diffusing out of the threads into the buffer, thus concentrating the signal without cross-reaction. This critical aspect enabled to increase the number of simultaneous immunoassay per sample in a scalable manner, which was unavailable for conventional ELISA. As a demonstration, we chemically modified the thread material as a substrate for bioassays, demonstrated singleplex colorimetric immunoassays with seven types of cytokines without any signs of erroneous cross-reaction, which was also validated with highly sensitive fluorescence immunoassay. This high resistance against off-target binding was ultimately proven by our 3,7-multiplexed immunoassay results. We anticipate the number of multiplexing to easily increase by adding more threads or by decreasing the dimension of each cotton thread compartment when the overall particle size is concerned. We also determined the dynamic range for accurate measurement of biomolecule concentrations, offering a simple and sensitive route for the precise determination of the presence of analyte. To apply this platform to serum and other body fluids, further optimization of antibody crosslinking, incubating methods, and washing protocols will be made in the future. We expect that this method will not only lead to the introduction of new manufacturing methodologies for the development of low-cost multiplexing bioassay platforms but also give small laboratories or institutions in developing countries the opportunity to have access to precise molecular analysis.

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[37]See supplementary material at http://dx.doi.org/10.1063/1.4927590 for supporting experiment results and details about fiber composite fabrication methodology.